THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Appellant(s):	M. Secretin
Appl. No.:	10/564,805
Conf. No.:	3416
Filed:	May 17, 2006
Title:	INFANT OR FOLLOW-ON FORMULA
Art Unit:	1782
Examiner:	Preston Smith
Docket No.:	3712036-00701

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

APPELLANTS' APPEAL BRIEF

Sir:

Appellants submit this Appeal Brief in support of the Notice of Appeal filed on April 21, 2011. This Appeal is taken from the Final Rejection in the Office Action dated January 19, 2011.

I. REAL PARTIES IN INTEREST

The real party in interest for the above-identified patent application on Appeal is Nestec S.A., by virtue of an Assignment recorded on November 21, 2006 at reel 018559, frames 0214-0216, in the United States Patent and Trademark Office.

II. RELATED APPEALS AND INTERFERENCES

Appellants' legal representative and the Assignees of this patent application do not know of any prior or pending appeals, interferences or judicial proceedings that may be related to, directly affect or be directly affected by or have a bearing on the Board's decision with respect to the above-identified Appeal.

III. STATUS OF CLAIMS

Claims 1 and 5-21 are rejected in this application. Claims 2-4 were previously canceled. Therefore, Claims 1 and 5-21 are being appealed in this Brief. A copy of the appealed claims is included in the Claims Appendix.

IV. STATUS OF AMENDMENTS

A Non-Final Office Action was mailed on December 22, 2009 rejecting the claims as obvious under 35 U.S.C. §103. Appellants responded to the Non-Final Office Action on March 25, 2010 arguing against the obviousness rejections and amending the claims. A Final Office Action was mailed on June 15, 2010 maintaining the obviousness rejections. Appellants responded to the Final Office Action on December 15, 2010 arguing against the obviousness rejections without further amending the claims. A Final Office Action was mailed on January 19, 2011 maintaining the obviousness rejections. Appellants filed a Notice of Appeal on April 21, 2011 in response to the Final Office Action.

V. SUMMARY OF CLAIMED SUBJECT MATTER

A summary of the claimed subject matter by way of reference to the specification and/or figures for each of the independent claims is provided as follows:

Independent Claim 1 recites an infant or follow-on formula (page 1, lines 5-9; page 2 lines 8-11; page 2, line 35 to page 3, line 12) comprising a source of proteins (page 2 lines 8-11; page 6, line 32 to page 7, line 29), a source of lipids (page 2 lines 8-11), a source of carbohydrates (page 2 lines 8-11) and a probiotic (page 2 lines 8-11; page 3, lines 14-16; page 6, lines 5-30) wherein the source of lipids comprises ARA and DHA (page 4, lines 19-26; page 4, line 28 to page 5, line 2), the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source (page 4, lines 19-26).

Independent Claim 14 recites a method for strengthening natural immune defenses of an infant or a baby (page 1, lines 5-9; page 2, lines 13-15) comprising feeding said infant or baby a formula (page 2, lines 13-15) comprising a source of proteins (page 2 lines 8-11; page 6, line 32 to page 7, line 29), a source of lipids (page 2 lines 8-11), a source of carbohydrates (page 2 lines 8-11) and a probiotic (page 2 lines 8-11; page 3, lines 14-16; page 6, lines 5-30) wherein the source of lipids comprises ARA and DHA (page 4, lines 19-26; page 4, line 28 to page 5, line 2), the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source (page 4, lines 19-26).

Independent Claim 16 recites a method of reducing at least one of flatulence, vomiting, regurgitation and/or morbidity (page 3, lines 18-25) by administering to a baby or infant a composition (page 3, lines 18-25) comprising a source of proteins (page 2 lines 8-11; page 6, line 32 to page 7, line 29), a source of lipids (page 2 lines 8-11), a source of carbohydrates (page 2 lines 8-11) and a probiotic (page 2 lines 8-11; page 3, lines 14-16; page 6, lines 5-30) wherein the source of lipids comprises ARA and DHA (page 4, lines 19-26; page 4, line 28 to page 5, line 2), the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source (page 4, lines 19-26).

Independent Claim 17 recites a method for promoting healthy mental development in an infant or a baby (page 1, lines 5-9; page 2, lines 17-19) comprising feeding said infant or baby a formula (page 2, lines 17-19) comprising a source of proteins (page 2 lines 8-11; page 6, line 32 to page 7, line 29), a source of lipids (page 2 lines 8-11), a source of carbohydrates (page 2 lines

8-11) and a probiotic (page 2 lines 8-11; page 3, lines 14-16; page 6, lines 5-30) wherein the source of lipids comprises ARA and DHA (page 4, lines 19-26; page 4, line 28 to page 5, line 2), the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source (page 4, lines 19-26), and wherein at least 40% of the proteins are modified sweet whey proteins with reduced CGMP (page 7, lines 15-29).

Although specification citations are given in accordance with 37 C.F.R. §1.192(c), these reference numerals and citations are merely examples of support in the specification for the terms used in this section of the Brief. There is no intention to suggest in any way that the terms of the claims are limited to the examples in the specification. As demonstrated by the references numerals and citations, the claims are fully supported by the specification as required by law. However, it is improper under the law to read limitations from the specification into the claims. Pointing out specification support for the claim terminology in accordance with Rule 1.192(c) does not in any way limit the scope of the claims to those examples from which they find support. Nor does this exercise provide a mechanism for circumventing the law precluding reading limitations into the claims from the specification. In short, the reference numerals and specification citations are not to be construed as claim limitations or in any way used to limit the scope of the claims.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1. Claims 1, 5-6, 9 and 14-16 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 6,306,908 to Carlson et al. ("*Carlson*") in view of U.S. Patent No. 5,902,578 to Halpin-Dohnalek et al. ("*Halpin-Dohnalek*") as evidenced by Bifidobacterial NPL ("*Bifidobacterial NPL*") and DHA NPL ("*DHA NPL*").

2. Claim 7 is rejected under 35 U.S.C. §103(a) as being unpatentable over *Carlson* in view of *Halpin-Dohnalek* and further in view of Effect of Bifidobacterium longum BB536 yogurt administration on the intestinal environment of healthy adults by T. Ogata et al. ("*Ogata*") as evidence by *Bifidobacterial NPL.*

3. Claim 8 is rejected under 35 U.S.C. §103(a) as being unpatentable over *Carlson* in view of *Halpin-Dohnalek* and further in view of EP 0904784 to Van Hoey-De-Boer et al. ("*Van Hoey-De-Boer*") as evidenced by *Bifidobacterial NPL.*

4. Claim 10 is rejected under 35 U.S.C. §103(a) as being unpatentable over *Carlson* in view of *Halpin-Dohnalek* and further in view of *Van Hoey-De-Boer* and *Ogata* as evidenced by *Bifidobacterial NPL.*

5. Claims 11-13 and 17-21 are rejected under 35 U.S.C. §103(a) as being unpatentable over *Carlson* in view of *Halpin-Dohnalek* and further in view of U.S. Patent No. 6,777,391 to Kratky et al. ("*Kratky*") as evidence by Threonine NPL ("*Threonine NPL*") and *Bifidobacterial NPL.*

VII. ARGUMENTS

A. LEGAL STANDARDS

Obviousness under 35 U.S.C. §103

The Federal Circuit has held that the legal basis for a determination of obviousness under 35 U.S.C. § 103 is:

> whether the claimed invention as a whole would have been obvious to a person of ordinary skill in the art at the time the invention was made...The foundational facts for the prima facie case of obviousness are: (1) the scope and content of the prior art; (2) the difference between the prior art and the claimed invention; and (3) the level of ordinary skill in the art...Moreover, objective indicia such as commercial success and long felt need are relevant to the determination of obviousness...Thus, each obviousness determination rests on its own facts.

In re Mayne, 41 U.S.P.Q. 2d 1451, 1453 (Fed. Cir. 1997).

In making this determination, the Examiner has the initial burden of proving a *prima facie* case of obviousness. *In re Rijckaert*, 9 F.3d 1531, 1532, 28 U.S.P.Q. 2d 1955, 1956 (Fed. Cir. 1993). This burden may only be overcome "by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings." *In re Fine*, 837 F.2d 1071, 1074, 5 U.S.P.Q. 2d 1596, 1598 (Fed. Cir. 1988). "If the examination at the initial stage does not produce a prima facie case of unpatentability, then without more the applicant is entitled to grant of the patent." *In re Oetiker*, 24 U.S.P.Q. 2d 1443, 1444 (Fed. Cir. 1992).

Moreover, the Examiner must provide explicit reasons why the claimed invention is obvious in view of the prior art. The Supreme Court has emphasized that when formulating a rejection under 35 U.S.C. § 103(a) based upon a combination of prior art elements it remains necessary to identify the reason why a person of ordinary skill in the art would have combined the prior art elements in the manner claimed. *KSR v. Teleflex*, 127 S. Ct. 1727 (2007).

Of course, references must be considered as a whole and those portions teaching against or away from the claimed invention must be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve Inc.*, 796 F.2d 443 (Fed. Cir. 1986). "A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference would be discouraged

from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the Applicant." *Monarch Knitting Mach. Corp. v. Fukuhara Indus. Trading Co., Ltd.*, 139 F.3d 1009 (Fed. Cir. 1998) (quoting *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994)).

B. THE CLAIMED INVENTION

There are four independent claims on appeal: Claims 1, 14 and 16-17. Independent Claim 1 recites an infant or follow-on formula comprising a source of proteins, a source of lipids, a source of carbohydrates and a probiotic wherein the source of lipids comprises ARA and DHA. The DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source.

Independent Claim 14 recites a method for strengthening natural immune defenses of an infant or a baby comprising feeding said infant or baby a formula comprising a source of proteins, a source of lipids, a source of carbohydrates and a probiotic wherein the source of lipids comprises ARA and DHA. The DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source.

Independent Claim 16 recites a method of reducing at least one of flatulence, vomiting, regurgitation and/or morbidity by administering to a baby or infant a composition comprising a source of proteins, a source of lipids, a source of carbohydrates and a probiotic wherein the source of lipids comprises ARA and DHA. The DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source.

Independent Claim 17 recites a method for promoting healthy mental development in an infant or a baby comprising feeding said infant or baby a formula comprising a source of proteins, a source of lipids, a source of carbohydrates and a probiotic. The source of lipids comprises ARA and DHA. The DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source. At least 40% of the proteins are modified sweet whey proteins with reduced CGMP.

According to the Commission Directive 91/321/EEC of 14 May 1991 on infant formulae and follow-on formulae, article 1.2(a), the term "infants" means children under the age of 12 months. This definition is adopted in the present specification. According to the Commission Directive 91/321/EEC of 14 May 1991 on infant formula and follow-on formula, article 1.2(c), the term "infant formula" means foodstuffs intended for particular nutritional use by infants

during the first four to six months of life and satisfying by themselves the nutritional requirements of this category of persons. This definition is adopted in the present specification. It is understood that infants can be fed solely with infant formulas, or that the infant formula can be as a complement of human milk.

According to the Commission Directive 91/321/EEC of 14 May 1991 on infant formulae and follow-on formulae, article 1.2(d), the term "follow-on formula" means foodstuffs intended for particular nutritional use by infants aged over four months and constituting the principal liquid element in a progressively diversified diet of this category of persons. This definition is adopted in the present specification.

Appellants have surprisingly found that feeding infants the formula of the present claims generally results in the promotion of the immune defenses of the infant as has been demonstrated by an enhanced response to vaccinations and/or improved gut barrier function and lower levels of intolerance of cows' milk protein coupled with satisfactory physical development. These results are summarized in Examples 1 and 2 of the specification where infants that were fed a formula according to the present claims were compared to infants that were fed a similar formula but without probiotics. The results demonstrate that infants fed the formulas of the present claims generally display strengthened immune defenses as demonstrated by an enhanced response to vaccinations and/or improved gut barrier function and lower levels of intolerance of cows' milk protein coupled with satisfactory physical development when compared with the control group. See US 2007/0031537, Examples at paragraphs [0069] – [0077].

Appellants previously submitted the methodology and trials for certain *in vitro* testing of certain exemplary compositions of the present claims (attached as Exhibit A). The methodology and trials detailed *in vitro* testing of compositions including ARA, DHA and *Lactobacillus paracasei* NCC 2461 (ST11). While *Lactobacillus paracasei* was selected for the present *in vitro* studies, Appellants submit that the presently claimed subject matter should not be limited to this probiotic. Generally, in the *in vitro* study, T84 cells were incubated overnight in serum free DMEM/F12 followed by a two hour pre-incubation with ST11 cells in the presence or absence of DHA/ARA. After the two hour pre-incubation, *Clostridium difficile* toxin A was added to the apical chamber. After an overnight incubation, transepithelial electrical resistances (TEER) were measured, and the protection of ingredients, alone and in combination, were measured. The

results of the test indicate that a combination of a probiotic and DHA/ARA provides better results than either ingredient alone.

Finally, formulas according to the present claims comprise DHA and ARA. High amounts of DHA alone, or use of DHA sources providing high levels of EPA, a fatty acid precursor of DHA, may however lead to depletion of the arachidonic status. Thus, DHA in formulas according to the present claims can preferably be provided by a low EPA fish oil at a level that has been shown to achieve DHA levels in the various blood pools of formula-fed infants similar to those of breast-fed infants. Accordingly, the claimed DHA content ranges between 0.2 and 0.5% of total fatty acids in the lipid source.

C. THE REJECTION OF CLAIMS 1, 5-6, 9 AND 14-16 UNDER 35 U.S.C. §103(A) TO *CARLSON, HALPIN-DOHNALEK, BIFIDOBACTERIAL NPL* AND *DHA NPL* SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS

1. *Carlson, Halpin-Dohnalek, Bifidobacterial NPL* and *DHA NPL* alone or in combination fail to disclose each and every element of independent Claims 16 and 45-46.

Independent Claim 1 recites, in part, infant or follow-on formulas comprising a probiotic and a source of lipids comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty acids in the lipid source. Independent Claims 14 and 16 recite, in part, methods comprising administering to an infant an infant or follow-on formula comprising a probiotic and a source of lipids comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty acids in the lipid source.

Carlson, Halpin-Dohnalek, Bifidobacterial NPL and *DHA NPL* alone or in combination fail to disclose or suggest each and every element of independent Claims 1, 14 and 16. *Carlson, Halpin-Dohnalek, Bifidobacterial NPL* and *DHA NPL* alone or in combination fail to disclose or suggest infant or follow-on formulas comprising a source of lipid comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty acids in the lipid source as required by independent Claims 1, 14 and 16. *Carlson, Halpin-Dohnalek, Bifidobacterial NPL*

and *DHA NPL* alone or in combination also fail to disclose or suggest infant or follow-on formulas comprising a probiotic and a source of lipid comprising ARA and DHA in the same formulas as required by independent Claims 1, 14 and 16.

Carlson discloses enteral formulas that contain long-chain polyunsaturated fatty acids (PUFAs), e.g., arachidonic acid (AA), and docosahexaenoic acid (DHA), essentially free of cholesterol. The enteral formulas are used in methods for reducing the incidence of necrotizing enterocolitis. *Carlson* fails to disclose or suggest the claimed DHA content and fails to disclose a probiotic anywhere in his specification.

The Examiner cites *Carlson* as teaching DHA and alleges that "[a]though *Carlson* fails to explicitly teach arachidonic acid and docosahexaenoic acid both being present in the formula wherein the docosahexaenoic acid amount is 0.2-0.5%, *Carlson* does teach that the amount of docosahexaenoic acid may range from 0.25-35 mg...[i]n light of these teachings, one of ordinary skill in the art would have found it obvious to slightly increase the docosahexaenoic acid content to 7 mg...to a slightly higher amount in order to boost the brain health boosting properties (produce known effects) of the formula (see docosahexaenoic acid NPL). Also, in light of the teachings discussed previously, the claimed range would have been discoverable by routine experimentation by one of ordinary skill in the art seeking to boost the brain health enhancing properties of the 'formula'." See Office Action, page 3, lines 10-20.

Appellants respectfully submit that, besides failing to disclose or suggest the range of DHA content in the total fatty acids as claimed, *Carlson* does not even mention any percentages or the requirement of specific percentages of any specific fatty acids (e.g., ARA and DHA) in the total fatty acids in the lipid source according to the present claims. Indeed, even if *Carlson* discloses one embodiment of a fatty acid profile having ARA and DHA, it is not proper for the Examiner to extrapolate weight percents of ARA and DHA in different embodiments because total weight percents change based on the compositions in the lipid profile.

Halpin-Dohnalek discloses a method for the prevention of infectious diarrhea or diarrhea caused by antibiotic therapy. The method involves mixing a powder comprising viable cultures of the probiotic organisms Lactobacillus reuteri, Lactobacillus acidophilus and Bifidobacierium infantis with a liquid and enterally administering the mixture to a mammal or a human. *Halpin-Dohnalek* fails to disclose or suggest any probiotic and ARA/DHA mixtures as *Halpin-Dohnalek* fails to even disclose the use of ARA/DHA. *Bifidobacterial NPL* and *DHA NPL* disclose

probiotics and DHA, respectively, but no combination of both in the same formula or composition.

For at least the reasons discussed above, *Carlson*, *Halpin-Dohnalek*, *Bifidobacterial NPL* and *DHA NPL* fail to disclose or suggest each and every element of independent Claims 1, 14 and 16. As a result, Appellants respectfully submit that independent Claims 1, 14 and 16, along with any claims that depend from Claims 1, 14 and 16, are novel and distinguishable from the cited references.

2. The Examiner has failed to rebut Appellant's evidence of unexpected result

"One way for a patent applicant to rebut a prima facie case of obviousness is to make a showing of 'unexpected results,' i.e., to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected." *In re Soni*, 54 F.3d 746, 750 (Fed. Cir. 1995). Appellants have surprisingly found that the combination of ingredients in the infant or follow-on formulas achieve unexpected results and are not disclosed in the cited references. In this regard, Appellants respectfully submit these surprising results to rebut a *prima facie* case of obviousness for at least the reasons set forth herein.

Appellants have surprisingly found that feeding infants the formula of the present claims generally results in the promotion of the immune defenses of the infant as has been demonstrated by an enhanced response to vaccinations and/or improved gut barrier function and lower levels of intolerance of cows' milk protein coupled with satisfactory physical development. These results are summarized in Examples 1 and 2 of the specification where infants that were fed a formula according to the present claims were compared to infants that were fed a similar formula but without probiotics. The results demonstrate that infants fed the formulas of the present claims generally display strengthened immune defenses as demonstrated by an enhanced response to vaccinations and/or improved gut barrier function and lower levels of intolerance of cows' milk protein coupled with satisfactory physical development when compared with the control group. See US 2007/0031537, Examples at paragraphs [0069] – [0077].

Appellants also submitted the methodology and trials detailing *in vitro* testing of compositions including ARA, DHA and *Lactobacillus paracasei* NCC 2461 (ST11). Generally,

in the *in vitro* study, T84 cells were incubated overnight in serum free DMEM/F12 followed by a two hour pre-incubation with ST11 cells in the presence or absence of DHA/ARA. After the two hour pre-incubation, *Clostridium difficile* toxin A was added to the apical chamber. After an overnight incubation, transepithelial electrical resistances (TEER) were measured, and the protection of ingredients, alone and in combination, were measured. The results of the test indicate that a combination of a probiotic and DHA/ARA provides better results than either ingredient alone.

Because Appellants have shown that administering a probiotic and a source of lipid comprising ARA and DHA in the same formulas provides advantages over diets not providing this combination of ingredients, Appellants have shown that the claimed invention provides unexpected results over the prior art. Accordingly, the showing of unexpected results provides evidence that the claimed invention is not *prima facie* obvious in view of the cited references.

3. The skilled artisan would have no reason to combine the cited references in the absence of hindsight

Appellants respectfully submit that the skilled artisan would have no reason arrive at the claimed invention using the cited references in the absence of hindsight because the cited references are entirely directed to different food products utilizing varying ingredients for different intended purposes. Moreover, references must be considered as a whole and those portions teaching against or away from each other and/or the claimed invention must be considered. *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve Inc.*, 796 F.2d 443 (Fed. Cir. 1986). "A prior art reference may be considered to teach away when a person of ordinary skill, upon reading the reference would be discouraged from following the path set out in the reference, **or would be led in a direction divergent from the path that was taken by the Applicant**." *Monarch Knitting Machinery Corp. v. Fukuhara Industrial Trading Co., Ltd.*, 139 F.3d 1009 (Fed. Cir. 1998), quoting, *In re Gurley*, 27 F.3d 551 (Fed. Cir. 1994) (emphasis added).

Carlson discloses enteral formulas that contain long-chain polyunsaturated fatty acids (PUFAs), e.g., arachidonic acid (AA), and docosahexaenoic acid (DHA), essentially free of cholesterol. The enteral formulas are used in methods for reducing the incidence of necrotizing

enterocolitis. *Carlson* fails to disclose or suggest the claimed DHA content and fails to disclose a probiotic anywhere in his specification. In addition, rather than relating the compositions to the promotion of the immune system, *Carlson* relates to compositions for reducing the incidence of necrotising enterocolitis, which is a life-threatening condition that is generally only a risk factor for infants born prematurely. To address this, *Carlson* teaches administering ARA and DHA, preferably in the form of phospholipids, as these are believed to be more effective than the triglyceride form. See *Carlson*, column 2, lines 33-39 and column 2, lines 20-45.

Halpin-Dohnalek is entirely directed toward the use of a mixture of three different probiotic bacterial species including a *Lactobacillus reuteri*, a *Lactobacillus acidophilus* and a *Bifidobacterium infantis* to prevent infectious diarrhea. See *Halpin-Dohnalek*, Abstract. *Halpin-Dohnalek* teaches that the presence of all three of these strains is necessary to achieve the desired result. See *Halpin-Dohnalek,* column 4, lines 57-60. Although mention is made of infant formula, the treatment is primarily directed to older children, as may be seen from the clinical study described at Example II. In fact, these probiotics would not be suitable for infants since both *Lactobacillus reuteri* and *Lactobacillus acidophilus* produce D(-) lactic acid and their consumption by children under three, particularly infants, is not recommended by the World Health Organization. Further, as stated above, *Halpin-Dohnalek* fails to even mention the promotion of the immune system of infants.

In regards to the alleged combination of *Carlson* with *Halpin-Dohnalek*, Appellants previously submitted an article by Kankaanpää (attached as Exhibit B), which demonstrates that the skilled artisan would be deterred from combining probiotics and polyunsaturated fatty acids (PUFAs) in accordance with the present claims. Specifically, Kankaanpää states that "[a]s polyunsaturated fatty acids (PUFA) possess antimicrobial properties, they may deter the action of probiotics" (emphasis added). See Kankaanpää, Abstract. Kankaanpää also states that "physiologically relevant levels of free PUFA may influence the functions of probiotics. Consequently, non-adhered probiotics may be washed out from the gastrointestinal tract and potential health benefits may be compromised" (emphasis added). See Kankaanpää, page 153. Appellants respectfully submit, therefore, that the Kankaanpää reference would have discouraged the skilled artisan from combining probiotics and PUFA to arrive at the present claims. Therefore, the skilled artisan would have no reason to combine at least *Carlson* with *Halpin-Dohnalek* in view of the Kankaanpää reference.

The Examiner asserts that "[t]he Kankaanpää reference does not appear to establish that combining probiotics with PUFA's would be disadvantageous in the references cited by [the] examiner." See Office Action, page 14, lines 3-8. Appellants submit that although the Kankaanpää reference may not compare specific compositions recited in the cited references, Kankaanpää does teach the skilled artisan that compositions having PUFA's and probiotics may not be beneficial in combination in compositions.

As the compositions and methods of the cited references are directed toward completely unrelated products having completely unrelated objectives, the skilled artisan would have no reason to combine the cited references to arrive at the present claims in the absence of hindsight. Indeed, the skilled artisan would not arrive at the present claims by reviewing such cited references having widely varying applications and entirely different objectives. Further, if the proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there exists no reason for the skilled artisan to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984).

Appellants also respectfully submit that the Examiner has applied hindsight reasoning by attempting to selectively piece together teachings of each of the references in an attempt to recreate what the claimed invention discloses. The Examiner alleges, however, that as long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the Appellant's disclosure, such a reconstruction is proper. See Office Action, page 14, lines 15-22. However, as evidenced by the Kankaanpää reference, the knowledge that was within the level of ordinary skill would at the time of the claimed invention establishes that the skilled artisan would be discouraged from combining probiotics and PUFAs, such as ARA and DHA, to arrive at the present claims. With that knowledge, it is clear that the Examiner applied hindsight reasoning by gleaning knowledge from Appellants' disclosure over that which was known at the time of the claimed invention (e.g., the Kankaanpää reference).

In sum, the skilled artisan would have no reason to arrive at the claimed invention using the cited references in the absence of hindsight. Therefore, the Examiner has not shouldered the burden of establishing a prima facie case of obviousness. The Examiner bears the initial burden of establishing a prima facie case. *In re Oetiker,* 977 F.2d 1443, 1445 (Fed. Cir. 1992). It is well settled that "rejections on obviousness grounds cannot be sustained by mere conclusory

statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *In re Kahn,* 441 F.3d 977, 988 (Fed. Cir. 2006). It thus follows that the Examiner's stated rejection of Claims 1, 14 and 16 does not satisfy the Examiner's initial burden of presenting evidence to establish a prima facie case of obviousness so as to shift the burden to Appellants to establish non-obviousness of the claimed invention. Accordingly, Appellants respectfully submit that the present claims are novel, nonobvious and distinguishable from the cited references and in condition for allowance.

D. THE REJECTION OF CLAIM 7 UNDER 35 U.S.C. §103(A) IN VIEW OF *CARLSON, HALPIN-DOHNALEK, OGATA* AND *BIFIDOBACTERIAL NPL* SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS

Appellants respectfully submit that the patentability of Claim 1 as previously discussed renders moot the obviousness rejection of Claim 7 that depends from Claim 1. In this regard, the cited art fails to teach or suggest the elements of Claim 7 in combination with the novel elements of Claim 1. For example, *Carlson, Halpin-Dohnalek, Ogata* and *Bifidobacterial NPL* alone or in combination fail to disclose or suggest infant or follow-on formulas comprising a source of lipid comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty acids in the lipid source as is required by independent Claim 1. *Carlson, Halpin-Dohnalek, Ogata* and *Bifidobacterial NPL* alone or in combination also fail to disclose or suggest infant or follow-on formulas comprising a probiotic and a source of lipid comprising ARA and DHA as required by independent Claim 1. Accordingly, Appellants respectfully submit that Claim 1, along with Claim 7 that depends from Claim 1, are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

Regarding the additional secondary reference, *Ogata* is entirely directed toward the effects of *Bifidobacterium longum* BB536 yogurt on the intestinal environment of healthy adults. See *Ogata*, Abstract. Although *Ogata* mentions the use of *Bifidobacterium longum* BB536, the article is entirely directed toward consumption by healthy adults and fails to even mention administration of BB536 to infants to strengthen their immune systems. As a result, *Ogata* would not even be considered by one skilled in the art that is seeking to improve the immune system of infants.

E. THE REJECTION OF CLAIM 8 UNDER 35 U.S.C. §103(A) IN VIEW OF *CARLSON, HALPIN-DOHNALEK, VAN HOEY-DE-BOER* AND *BIFIDOBACTERIAL NPL* SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS

Appellants respectfully submit that the patentability of Claim 1 as previously discussed renders moot the obviousness rejection of Claim 8 that depends from Claim 1. In this regard, the cited art fails to teach or suggest the elements of Claim 8 in combination with the novel elements of Claim 1. For example, *Carlson*, *Halpin-Dohnalek*, *Van Hoey-De-Boer* and *Bifidobacterial NPL* alone or in combination fail to disclose or suggest infant or follow-on formulas comprising a source of lipid comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty acids in the lipid source as is required by independent Claim 1. *Carlson*, *Halpin-Dohnalek*, *Van Hoey-De-Boer* and *Bifidobacterial NPL* alone or in combination also fail to disclose or suggest infant or follow-on formulas comprising a probiotic and a source of lipid comprising ARA and DHA as required by independent Claim 1. Accordingly, Appellants respectfully submit that Claim 1, along with Claim 8 that depends from Claim 1, are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

Regarding the additional secondary reference, *Van Hoey-De-Boer* is entirely related to a nutritional composition containing a minimum of three different probiotic strains with the intention of providing protection against infection all the way along the gastro-intestinal tract, thus obviating the need to identify the type of micro-organism responsible for the infection. See *Van Hoey-De-Boer,* Abstract. The benefits of such compositions include, for example, therapy or prophylaxis of multiple disorders of the gastrointestinal tract such as IBS, Crohn's disease and cancer of the GI tract. However, *Van Hoey-De-Boer* fails to even suggest that probiotics may play a useful role in strengthening the immune system of infants as claimed.

F. THE REJECTION OF CLAIM 10 UNDER 35 U.S.C. §103(A) IN VIEW OF *CARLSON, HALPIN-DOHNALEK, VAN HOEY-DE-BOER, OGATA* AND *BIFIDOBACTERIAL NPL* SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS

Appellants respectfully submit that the patentability of Claim 1 as previously discussed renders moot the obviousness rejection of Claim 10 that depends from Claim 1. In this regard, the cited art fails to teach or suggest the elements of Claim 10 in combination with the novel elements of Claim 1. For example, *Carlson, Halpin-Dohnalek, Van Hoey-De-Boer, Ogata* and *Bifidobacterial NPL* alone or in combination fail to disclose or suggest infant or follow-on formulas comprising a source of lipid comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty acids in the lipid source as is required by independent Claim 1. *Carlson, Halpin-Dohnalek, Van Hoey-De-Boer, Ogata* and *Bifidobacterial NPL* alone or in combination also fail to disclose or suggest infant or follow-on formulas comprising a probiotic and a source of lipid comprising ARA and DHA as required by independent Claim 1. Accordingly, Appellants respectfully submit that Claim 1, along with Claim 10 that depends from Claim 1, are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

Regarding the additional secondary references, *Van Hoey-De-Boer* is entirely related to a nutritional composition containing a minimum of three different probiotic strains with the intention of providing protection against infection all the way along the gastro-intestinal tract, thus obviating the need to identify the type of micro-organism responsible for the infection. See *Van Hoey-De-Boer,* Abstract. The benefits of such compositions include, for example, therapy or prophylaxis of multiple disorders of the gastrointestinal tract such as IBS, Crohn's disease and cancer of the GI tract. However, *Van Hoey-De-Boer* fails to even suggest that probiotics may play a useful role in strengthening the immune system of infants as claimed.

Ogata is entirely directed toward the effects of *Bifidobacterium longum* BB536 yogurt on the intestinal environment of healthy adults. See *Ogata*, Abstract. Although *Ogata* mentions the

use of *Bifidobacterium longum* BB536, the article is entirely directed toward consumption by healthy adults and fails to even mention administration of BB536 to infants to strengthen their immune systems. As a result, *Ogata* would not even be considered by one skilled in the art that is seeking to improve the immune system of infants.

G. THE REJECTION OF CLAIMS 11-13 AND 17-21 UNDER 35 U.S.C. §103(A) IN VIEW OF *CARLSON, HALPIN-DOHNALEK, KRATKY, THREONINE NPL* AND *BIFIDOBACTERIAL NPL* SHOULD BE REVERSED BECAUSE THE EXAMINER HAS FAILED TO ESTABLISH A *PRIMA FACIE* CASE OF OBVIOUSNESS

Appellants respectfully submit that the patentability of Claim 1 as previously discussed renders moot the obviousness rejection of Claims 11-13 and 20 that depend from Claim 1. In this regard, the cited art fails to teach or suggest the elements of Claims 11-13 and 20 in combination with the novel elements of Claim 1. For example, *Carlson, Halpin-Dohnalek, Kratky, Threonine NPL* and *Bifidobacterial NPL* alone or in combination fail to disclose or suggest infant or follow-on formulas comprising a source of lipid comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty acids in the lipid source as is required by independent Claim 1. *Carlson, Halpin-Dohnalek, Kratky, Threonine NPL* and *Bifidobacterial NPL* alone or in combination also fail to disclose or suggest infant or follow-on formulas comprising a probiotic and a source of lipid comprising ARA and DHA as required by independent Claim 1. Accordingly, Appellants respectfully submit that Claim 1, along with Claims 11-13 and 20 that depend from Claim 1, are novel, nonobvious and distinguishable from the cited references and are in condition for allowance.

1. *Carlson, Halpin-Dohnalek, Kratky, Threonine NPL* and *Bifidobacterial NPL* alone or in combination fail to disclose each and every element of independent Claim 17.

Carlson, Halpin-Dohnalek, Kratky, Threonine NPL and *Bifidobacterial NPL* alone or in combination fail to disclose or suggest each and every element of independent Claim 17. *Carlson, Halpin-Dohnalek, Kratky, Threonine NPL* and *Bifidobacterial NPL* alone or in combination fail to disclose or suggest infant or follow-on formulas comprising a source of lipid comprising ARA and DHA, wherein the DHA content is between 0.2 and 0.5% of the total fatty

acids in the lipid source as required by independent Claim 17. *Carlson*, *Halpin-Dohnalek*, *Kratky*, *Threonine NPL* and *Bifidobacterial NPL* alone or in combination also fail to disclose or suggest infant or follow-on formulas comprising a probiotic and a source of lipid comprising ARA and DHA in the same formulas as required by independent Claim 17.

Carlson discloses enteral formulas that contain long-chain polyunsaturated fatty acids (PUFAs), e.g., arachidonic acid (AA), and docosahexaenoic acid (DHA), essentially free of cholesterol. The enteral formulas are used in methods for reducing the incidence of necrotizing enterocolitis. *Carlson* fails to disclose or suggest the claimed DHA content and fails to disclose a probiotic anywhere in his specification.

The Examiner cites *Carlson* as teaching DHA and alleges that "[a]though *Carlson* fails to explicitly teach arachidonic acid and docosahexaenoic acid both being present in the formula wherein the docosahexaenoic acid amount is 0.2-0.5%, *Carlson* does teach that the amount of docosahexaenoic acid may range from 0.25-35 mg...[i]n light of these teachings, one of ordinary skill in the art would have found it obvious to slightly increase the docosahexaenoic acid content to 7 mg...to a slightly higher amount in order to boost the brain health boosting properties (produce known effects) of the formula (see docosahexaenoic acid NPL). Also, in light of the teachings discussed previously, the claimed range would have been discoverable by routine experimentation by one of ordinary skill in the art seeking to boost the brain health enhancing properties of the 'formula'." See Office Action, page 3, lines 10-20.

Appellants respectfully submit that, besides failing to disclose or suggest the range of DHA content in the total fatty acids as claimed, *Carlson* does not even mention any percentages or the requirement of specific percentages of any specific fatty acids (e.g., ARA and DHA) in the total fatty acids in the lipid source according to the present claims. Indeed, even if *Carlson* discloses one embodiment of a fatty acid profile having ARA and DHA, it is not proper for the Examiner to extrapolate weight percents of ARA and DHA in different embodiments because total weight percents change based on the compositions in the lipid profile.

Halpin-Dohnalek discloses a method for the prevention of infectious diarrhea or diarrhea caused by antibiotic therapy. The method involves mixing a powder comprising viable cultures of the probiotic organisms Lactobacillus reuteri, Lactobacillus acidophilus and Bifidobacieriurn infantis with a liquid and enterally administering the mixture to a mammal or a human. *Halpin-*

Dohnalek fails to disclose or suggest any probiotic and ARA/DHA mixtures as *Halpin-Dohnalek* fails to even disclose the use of ARA/DHA.

Kratky discloses a composition comprising all of: i) acid whey protein or sweet whey protein from which caseino-glyco-macropeptide has been removed; and ii) free arginine; and iii) free histidine; and iv) free tyrosine or free tryptophan or tryptophan rich milk protein or a mixture thereof. *Kratky* fails to disclose combinations of probiotics and lipids in the same formula or composition or the recited DHA content. *Threonine NPL* fails to disclose or suggest any probiotics and ARA/DHA. *Bifidobacterial NPL* discloses probiotics but no combination of probiotics and lipids in the same formula or composition.

For at least the reasons discussed above, *Carlson*, *Halpin-Dohnalek*, *Kratky*, *Threonine NPL* and *Bifidobacterial NPL* fail to disclose or suggest each and every element of independent Claim 17. As a result, Appellants respectfully submit that independent Claim 17, along with any claims that depend from Claim 17, are novel and distinguishable from *Carlson*, *Halpin-Dohnalek*, *Kratky*, *Threonine NPL* and *Bifidobacterial NPL*.

2. The skilled artisan has no reason to combine the cited references in the absence of hindsight

Appellants respectfully submit that the skilled artisan would have no reason arrive at the claimed invention using the cited references in the absence of hindsight because the cited references are entirely directed to different food products utilizing varying ingredients for different intended purposes. *Carlson* discloses enteral formulas that contain long-chain polyunsaturated fatty acids (PUFAs), e.g., arachidonic acid (AA), and docosahexaenoic acid (DHA), essentially free of cholesterol. Rather than relating the compositions to the promotion of the immune system, *Carlson* relates to compositions for reducing the incidence of necrotising enterocolitis, which is a life-threatening condition that is generally only a risk factor for infants born prematurely. To address this, *Carlson* teaches administering ARA and DHA, preferably in the form of phospholipids, as these are believed to be more effective than the triglyceride form. See *Carlson*, column 2, lines 33-39 and column 2, lines 20-45.

Halpin-Dohnalek is entirely directed toward the use of a mixture of three different probiotic bacterial species including a *Lactobacillus reuteri*, a *Lactobacillus acidophilus* and a

Bifidobacterium infantis to prevent infectious diarrhea. See *Halpin-Dohnalek*, Abstract. *Halpin-Dohnalek* teaches that the presence of all three of these strains is necessary to achieve the desired result. See *Halpin-Dohnalek,* column 4, lines 57-60. Although mention is made of infant formula, the treatment is primarily directed to older children, as may be seen from the clinical study described at Example II. In fact, these probiotics would not be suitable for infants since both *Lactobacillus reuteri* and *Lactobacillus acidophilus* produce D(-) lactic acid and their consumption by children under three, particularly infants, is not recommended by the World Health Organization. Further, as stated above, *Halpin-Dohnalek* fails to even mention the promotion of the immune system of infants.

As previously discussed, Kankaanpää demonstrates that the skilled artisan would be deterred from combining probiotics and polyunsaturated fatty acids (PUFAs), as is required, in part, by the present claims. Specifically, Kankaanpää states that "[a]s polyunsaturated fatty acids (PUFA) possess antimicrobial properties, they may deter the action of probiotics" (emphasis added). See Kankaanpää, Abstract. Kankaanpää also states that "physiologically relevant levels of free PUFA may influence the functions of probiotics. Consequently, non-adhered probiotics may be washed out from the gastrointestinal tract and potential health benefits may be compromised" (emphasis added). See, Kankaanpää, page 153. Appellants respectfully submit, therefore, that the Kankaanpää reference would have discouraged the skilled artisan from combining probiotics and PUFA to arrive at the present claims. Therefore, the skilled artisan would have no reason to combine at least *Carlson* with *Halpin-Dohnalek* in view of the Kankaanpää reference.

Kratky is entirely directed toward an infant formula having a low threonine content and whey protein. See *Kratky*, column 1, lines 4-25. *Kratky* fails to disclose any polyunsaturated fatty acids, including ARA and DHA, in combination with any probiotics or the benefits of same for strengthening the immune system of infants.

Because the compositions and methods of the cited references are directed toward completely unrelated products having completely unrelated objectives, the skilled artisan would have no reason to combine the cited references to arrive at the present claims in the absence of hindsight. Indeed, the skilled artisan would not arrive at the present claims by reviewing such cited references having widely varying applications and entirely different objectives. Further, if the proposed modification would render the prior art invention being modified unsatisfactory for

its intended purpose, then there exists no reason for the skilled artisan to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984).

Appellants also respectfully submit that the Examiner has applied hindsight reasoning by attempting to selectively piece together teachings of each of the references in an attempt to recreate what the claimed invention discloses. The Examiner asserts, however, that as long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the Appellant's disclosure, such a reconstruction is proper. See Office Action, page 14, lines 15-22. However, as evidenced by the Kankaanpää reference, the knowledge that was within the level of ordinary skill would at the time of the claimed invention establishes that the skilled artisan would be discouraged from combining probiotics and PUFAs, such as ARA and DHA, to arrive at the present claims. With that knowledge, it is clear that the Examiner applied hindsight reasoning by gleaning knowledge from Appellants' disclosure over that which was known at the time of the claimed invention (e.g., the Kankaanpää reference).

In sum, the skilled artisan would have no reason to arrive at the claimed invention using the cited references in the absence of hindsight. Therefore, the Examiner has not shouldered the burden of establishing a prima facie case of obviousness. The Examiner bears the initial burden of establishing a prima facie case. *In re Oetiker,* 977 F.2d 1443, 1445 (Fed. Cir. 1992). It is well settled that "rejections on obviousness grounds cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness." *In re Kahn,* 441 F.3d 977, 988 (Fed. Cir. 2006). It thus follows that the Examiner's stated rejection of the present claims does not satisfy the Examiner's initial burden of presenting evidence to establish a prima facie case of obviousness so as to shift the burden to Appellants to establish non-obviousness of the claimed invention. Accordingly, Appellants respectfully submit that the present claims are novel, nonobvious and distinguishable from the cited references and in condition for allowance.

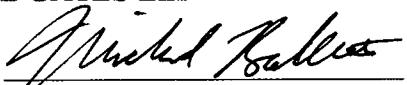
VIII. CONCLUSION

Appellants respectfully submit that the Examiner has failed to establish obviousness under 35 U.S.C. §103 with respect to the rejections of Claims 1 and 5-21. Accordingly, Appellants respectfully submit that the obviousness rejections are erroneous in law and in fact and should therefore be reversed by this Board.

A check in the amount of $510 is submitted herewith to cover the cost of the Appeal Brief. The Director is authorized to charge any additional fees that may be required, or to credit any overpayment to Deposit Account No. 02-1818. If such a withdrawal is made, please indicate the Attorney Docket No. 3712036-701 on the account statement.

Respectfully submitted,

K&L GATES LLP

BY 

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Dated: June 21, 2011

CLAIMS APPENDIX

PENDING CLAIMS ON APPEAL OF U.S. PATENT APPLICATION SERIAL NO. 10/564,805

1. Infant or follow-on formula comprising a source of proteins, a source of lipids, a source of carbohydrates and a probiotic wherein the source of lipids comprises ARA and DHA, the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source.

5. The formula according to Claim 1, wherein the ratio of ARA:DHA is between 0.8:1 and 1.2:1.

6. The formula according to Claim 1, wherein the probiotic is selected from the group consisting of a Bifidobacteria and a Lactobacillus.

7. The formula according to Claim 6, wherein the Bifidobacteria is Bifidobacterium longum BB 536.

8. The formula according to Claim 6, wherein the Lactobacillus is Lactobacillus paracasei rhamnosus GG.

9. The formula according to Claim 1, which contains both a Bifidobacterium and a Lactobacillus.

10. The formula according to Claim 9, wherein the Bifidobacteria is Bifidobacterium longum BB 536 and the Lactobacillus is Lactobacillus paracasei rhamnosus GG.

11. The formula according to Claim 1, wherein at least 40% of the proteins are modified sweet whey proteins with no CGMP or reduced CGMP.

12. The formula according to Claim 11, wherein the at least 60% of the proteins are modified sweet whey proteins comprising no CGMP or reduced CGMP.

13. The formula according to Claim 11, wherein the proteins are present in a maximum proportion of 2g/100 kcal.

14. Method for strengthening natural immune defenses of an infant or a baby comprising feeding said infant or baby a formula comprising a source of proteins, a source of lipids, a source of carbohydrates and a probiotic wherein the source of lipids comprises ARA and DHA, the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source.

15. The method according to Claim 14, wherein the formula provides the complete nutritional needs of the infant or baby.

16. Method of reducing at least one of flatulence, vomiting, regurgitation and/or morbidity by administering to a baby or infant a composition comprising a source of proteins, a

source of lipids, a source of carbohydrates and a probiotic wherein the source of lipids comprises ARA and DHA, the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source.

17. Method for promoting healthy mental development in an infant or a baby comprising feeding said infant or baby a formula comprising a source of proteins, a source of lipids, a source of carbohydrates and a probiotic wherein the source of lipids comprises ARA and DHA, the DHA content is between 0.2 and 0.5% of total fatty acids in the lipid source, and wherein at least 40% of the proteins are modified sweet whey proteins with reduced CGMP.

18. The method according to Claim 17, wherein the formula provides the complete nutritional needs of the baby or infant.

19. The method according to Claim 17, wherein the infant is preterm.

20. The formula according to Claim 11, wherein the proteins are present in a proportion of between 1.8 and 1.85 g/100 kcal.

21. The method according to Claim 17, wherein at least 40% of the proteins are modified sweet whey proteins with no CGMP.

EVIDENCE APPENDIX

Exhibit A: Methodology and trials for certain *in vitro* testing of certain exemplary compositions of the present claims

Exhibit B: Kankaanpää article

RELATED PROCEEDINGS APPENDIX

None

EXHIBIT A

Detailed note

Methodology and Trials:

Probiotic tested: Lactobacillus *paracasei* NCC 2461 (ST11), from Nestle Culture collection

Long Chain Poly Unsaturated Fatty Acids (PUFA) tested: Arachadonic acid (ARA) and Docosahexaenoic acid (DHA) Sigma Chemicals.

Cell culture: T84 cells were obtained from the American type culture collection (ATCC CCL-248). For detailed report on T84 cell culture amplification and seeding onto transwell inserts see R&D report-RD070106-Peter Duncan (NRC, Lausanne).

Preparation of ingredients: ST11 were grown in deMan Rogosa Sharpe (MRS; Difco) broth overnight at 37^0C. 100µl of the overnight culture of ST11/MRS was then grown again in 10 ml of MRS overnight at 37^0C. ST11 were then pelleted at 2000 rpm/2 mins, and washed in 10 ml PBS. To remove all traces of MRS, ST11 were spun again at 2000 rpm/ 2 mins, and the pellet resuspended in 2 ml of PBS. Calculation of cells/ml = O.D.600nM X cuvette dilution factor X 0.6*108 cells/ml.

PUFA albumin complexes were generated using the method described by Van Harken, Dixon, and Heimberg (J Biol Chem. 1969 May 10;244(9):2278-85). 14.7 mg of ARA and 15.76 mg DHA was dissolved with gentle heating in 4 ml of 0.9% NaCl. A 1g sample of BSA was also dissolved in 5 ml 0.9% NaCl, and the pH adjusted to 7.4. The warmed fatty acid solution was then added to the BSA solution and stirred overnight with N_2 gassing to expel oxygen (preventing oxidation and deteriation of ARA/DHA complex). The complex was then filter sterilized using a 0.45 µM filter. The final concentration of stock complex was 4.5 mM DHA/4.5mM ARA/1.5mM BSA.

In vitro model to test synergy: Inhibition of *Clostridium difficile* toxin A induced increase in barrier permeability in T84 epithelial cell monolayers. Permeability was assessed by measurement of the transepithelial electrical resistance (TEER).

Assay conditions: T84 cells were incubated overnight in serum free DMEM/F12 followed by a 2 hr pre-incubation with 10^3 to 10^4 ST11 bacteria cells/ml in the presence or absence of 11-44µM DHA/ARA added to the apical chamber. After the 2 hr pre-incubation, 100ng/ml *Clostridium difficile* toxin A was added to the apical chamber. After an overnight incubation (approx 15-18 hrs) TEERs were measured, and protection of ingredients alone and in combination measured.

Calculation of protection: TEERs were measured 2 hrs prior to *Clostridium difficile* toxin A addition. Variations in TEER were normalized to TEER 100% protection (namely wells containing 20% MRS). % protection (relative MRS) = $TEER_{(MRS)}$-$TEER_{(exp)}$ / $TEER_{(MRS)}$ -$TEER_{(no\ protection)}$ X 100

Statistical analysis: Each presented data point represents eight replicates measured in duplicates. A paired two tail distribution TTEST was performed using excel statistics package.

Results:

To test whether the selected ingredients, alone or in combination, protected T84 cells against Toxin A induced increase in barrier permeability, cells grown to a TEER > 2000 Ωcm^2 were incubated for 2hrs with 10^4 ST11 cells/ml and 22µM ARA/DHA, followed by an overnight exposure to 100ng/ml *C.difficile* Toxin A. TEERs were measured after 15-18 hr exposure to toxin A in the presence or absence of selected ingredients. The results are shown in the Figure below and it

may be seen that a combination of probiotic and DHA/ARA gave a better result than either ingredient alone.

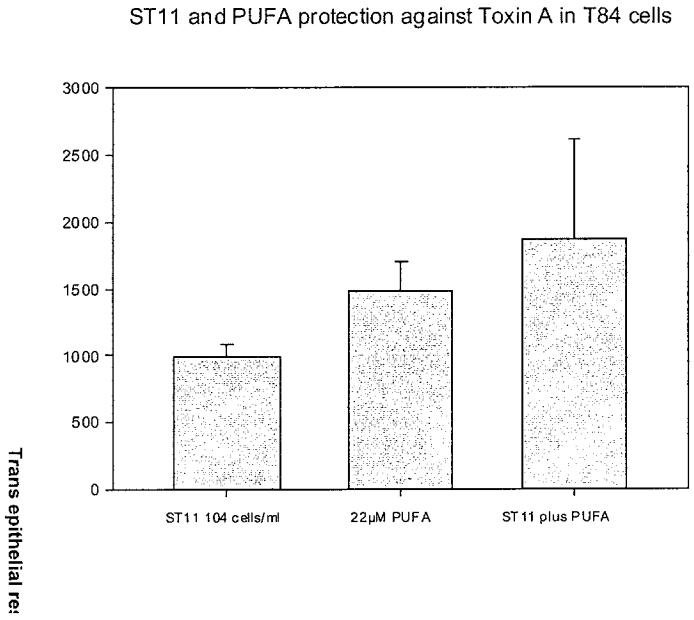

EXHIBIT B

ELSEVIER

FEMS Microbiology Letters 194 (2001) 149–153

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The influence of polyunsaturated fatty acids on probiotic growth and adhesion

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Abstract

The establishment of the intestinal microflora, and probiotic bacteria, may control the inflammatory conditions in the gut. As polyunsaturated fatty acids (PUFA) possess antimicrobial activities, they may deter the action of probiotics. We assessed whether free linoleic, γ-linolenic, arachidonic, α-linolenic and docosahexaenoic acids at physiological concentrations in the growth media would influence the growth and adhesion of *Lactobacillus* GG (probiotic), *Lactobacillus casei* Shirota (probiotic) and *Lactobacillus bulgaricus* (dairy strain). Higher concentrations of PUFA (10–40 μg PUFA ml^{-1}) inhibited growth and mucus adhesion of all tested bacterial strains, whilst growth and mucus adhesion of *L. casei* Shirota was promoted by low concentrations of γ-linolenic acid and arachidonic acid (at 5 μg ml^{-1}), respectively. PUFA also altered bacterial adhesion sites on Caco-2 cells. Caco-2 cells grown in the presence of arachidonic acid were less adhered to by all three bacterial strains. Yet, *L. casei* Shirota adhered better on Caco-2 cells grown in the presence of α-linolenic acid. As the adhesion to mucosal surfaces is pivotal in health promoting effects by probiotics, our results indicate that the action of probiotics in the gut may be modulated by dietary PUFA.

Keywords: Probiotic bacterium; Polyunsaturated fatty acid; Adhesion; Caco-2 cell; Intestinal mucus; *Lactobacillus*

1. Introduction

The modern hygiene conditions common in Western societies have been speculated to delay the development of the normal intestinal microflora [1]. This could be counteracted by the use of microbial cell preparations, so-called probiotics that are defined as live microbial food ingredients beneficial to health [2]. Probiotics possess well-documented efficacy in the prevention and treatment of diarrhoeal diseases [3,4] as well as modulating immune responses [5]. These beneficial effects have been explained by promotion of gut barrier functions.

However, other dietary components might also influence these gut functions. Dietary polyunsaturated fatty acids (PUFA) have been reported to influence epithelial cell membrane functions by modifying overall membrane fluidity, thickness, lipid-phase properties, microenvironment and interactions between fatty acids and membrane proteins [6]. In the context of microflora, PUFA have also been shown to possess antibacterial properties [7] and thus they could compromise the action of indigenous microflora or supplemented probiotics.

Dairy products, the most common delivery vehicle used to introduce probiotics, have a distinct texture and composition, and promote the passage of viable strains through acidic conditions in the stomach [8]. However, dairy products also contain PUFA and, thus, the question can be asked whether they are optimal carriers for probiotics? As the ability to permanently, or at least temporarily, adhere to intestinal mucosal surfaces appears to be an important aspect for optimal function of probiotics [2], we investigated the effects of different free PUFA in growth media on the adhesion properties of commonly used probiotics using two in vitro adhesion models; mucus and the Caco-2 cell adhesion. Moreover, the influence of PUFA on bacterial growth was assessed.

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0378-1097/01/$20.00
PII: S0378-1097(00)00519-X

2. Materials and methods

2.1. Micro-organisms

Lactic acid bacterial strains *Lactobacillus rhamnosus* strain GG (ATCC53103), *Lactobacillus delbrueckii* ssp. *bulgaricus* (ATCC11842) and *Lactobacillus casei* strain Shirota were selected as their in vitro adhesion properties have been previously reported [9,10]. Furthermore, *Lactobacillus* GG and *L. casei* Shirota have been commercially used in probiotic products and are the most thoroughly investigated probiotics, whereas *L. bulgaricus* is a commonly used dairy starter strain.

2.2. Bacterial growth

De Man, Rogosa, Sharpe (MRS) broth (Merck, Darmstadt, Germany) [11] was supplemented with or without linoleic (18:2 ω-6), γ-linolenic (18:3 ω-6), arachidonic (20:4 ω-6), α-linolenic (18:3 ω-3), or docosahexaenoic acid (22:6 ω-3) at final concentrations of 5, 10, 20 and 40 μg ml^{-1}. The range of PUFA concentrations used in this study has been previously demonstrated to be strain-dependently antibacterial [7,12] and physiologically relevant [13]. Bacteria were grown anaerobically in different MRS broths at 37°C with gentle agitation to facilitate mixing during the incubation period (18–24 h). The final bacterial concentrations and bacterial viability were assessed using the flow cytometric viability staining method based on bacterial membrane permeability [14].

2.3. Mucus adhesion assay

Bacteria were grown in MRS broths supplemented with the same PUFAs at 10, 20 and 40 μg ml^{-1} to the late exponential phase. Human intestinal mucus was isolated from the faeces of healthy infants ($n = 11$) and the bacterial adhesion to this mucus isolate was studied according to the method described by Kirjavainen et al. [9]. The results shown are the mean (S.D.) of three independent experiments performed in triplicate.

2.4. Caco-2 cells, culture conditions and adhesion assay

The Caco-2 cells (ATCC HTB 37) were cultured in minimal essential medium (MEM) supplemented with 1% MEM non-essential amino acids solution (Gibco BRL, NY, USA), 20% heat-inactivated (30 min, 56°C) foetal bovine serum, 2 mM L-glutamine, 2% sodium bicarbonat (7.5% NaH_2CO_2), 1% 0.1 M sodium pyruvate, 100 U ml^{-} penicillin and 100 mg ml^{-1} streptomycin at 37°C in an atmosphere of 10% CO_2/90% air.

Caco-2 monolayers were prepared in 24-well tissue culture plates, seeded at a concentration of $1–3 \times 10^5$ cells/well to obtain confluence, and maintained for 2 weeks (fresh medium changed every other day). To study the effects of PUFA on Caco-2 cells, MEM was supplemented with either linoleic acid, γ-linolenic acid, arachidonic acid, α-linolenic acid, or docosahexaenoic acid at a final concentration of 10 μg ml^{-1}. This PUFA concentration was selected according to preliminary growth and mucus ad-

Table 1
The effect of different PUFA on bacterial growth

PUFA	μg/ml	*Lactobacillus* GG (% final viable count)	*L. casei* Shirota (% final viable count)	*L. bulgaricus* (% final viable count)
Control	0	100	100	100
Linoleic acid (18:2 ω-6)	5	64	95	84
	10	79	62	77
	20	43	47	54
	40	8	7[a]	6[a]
γ-Linolenic acid (18:3 ω-6)	5	142	153[a]	105
	10	41	51[a]	41
	20	8[a]	18[a]	13
	40	5[a]	9[a]	6[a]
Arachidonic acid (20:4 ω-6)	5	138	98	156
	10	119	57	148
	20	59	45[a]	74
	40	12[a]	14[a]	8
α-Linolenic acid (18:3 ω-3)	5	36	55	81
	10	28	35[a]	43
	20	2[a]	10[a]	5[a]
	40	1[a]	6[a]	4[a]
Docosahexaenoic acid (22:6 ω-3)	5	58	78	63
	10	67	39	76
	20	75	67	62
	40	64	7[a]	45

% final viable count was determined by using flow cytometric method; the number of viable bacteria was enumerated after culturing with different free PUFA at 5, 10, 20 and 40 μg ml^{-1} and finally compared to that of the control.
[a]Significantly different from control (Student's *t*-test, $P < 0.05$).

hesion findings. One hour before bacterial adhesion assays, fresh non-supplemented MEM was changed.

Bacteria were grown in non-supplemented MRS broth to the late exponential phase. The adhesion of metabolically labelled bacteria to differentially cultured Caco-2 cells was assessed using the method described by Tuomola et al. [10]. The results presented are the mean (S.D.) of three independent experiments performed in triplicate.

2.5. Statistical analysis

The StatView 4.57 (Abacus Concepts, Berkeley, CA, USA) statistical program was used to analyse the data. A Student's *t*-test was used to determine significant differences ($P < 0.05$) in bacterial growth and adhesion in the presence or absence of PUFA.

3. Results

3.1. Bacterial growth

The effects of free PUFAs in the growth medium on bacterial growth are presented in Table 1. Linoleic acid inhibited the growth of *L. casei* Shirota and *L. bulgaricus* at 40 μg ml^{-1} ($P < 0.05$ and $P < 0.02$, respectively). γ-Linolenic acid suppressed the growth of *L. casei* Shirota at 10 μg ml^{-1} or higher ($P < 0.03$), *Lactobacillus* GG at 20 μg ml^{-1} or higher ($P < 0.01$) and *L. bulgaricus* at 40 μg ml^{-1} ($P < 0.05$), but promoted the growth of *L. casei* Shirota at 5 μg ml^{-1} ($P < 0.05$). Arachidonic acid inhibited the growth of *L. casei* Shirota at 20 μg ml^{-1} or higher ($P < 0.02$) and that of *Lactobacillus* GG at 40 μg ml^{-1} ($P < 0.01$). α-Linolenic acid suppressed the growth of *Lactobacillus* GG and *L. bulgaricus* at 20 μg ml^{-1} or higher ($P < 0.05$ and $P < 0.02$, respectively), whilst the growth of *L. casei* Shirota was already inhibited at 10 μg ml^{-1} ($P < 0.05$). Docosahexaenoic acid suppressed only the growth of *L. casei* Shirota at 40 μg ml^{-1} ($P < 0.01$).

Although PUFA at higher concentrations inhibited the growth of bacteria (seen as lowered % of final number of viable bacteria in Table 1), the viability of bacteria was not impaired. This was evinced by flow cytometric assessment of bacterial viability showing that over 95% of each bacterial strain possessed nonpermeable cell membrane to propidium iodide and were regarded as viable.

3.2. Mucus adhesion

The bacterial concentrations used did not saturate the binding capacity of mucus isolate. Generally, higher doses of PUFA in the growth medium inhibited bacterial adhesion to human intestinal mucus (Table 2). Linoleic acid in the growth media (40 μg ml^{-1}) inhibited the adhesion of *L. casei* Shirota ($P < 0.02$). Arachidonic and γ-linolenic acid at 20 μg ml^{-1} or higher in the growth media inhibited

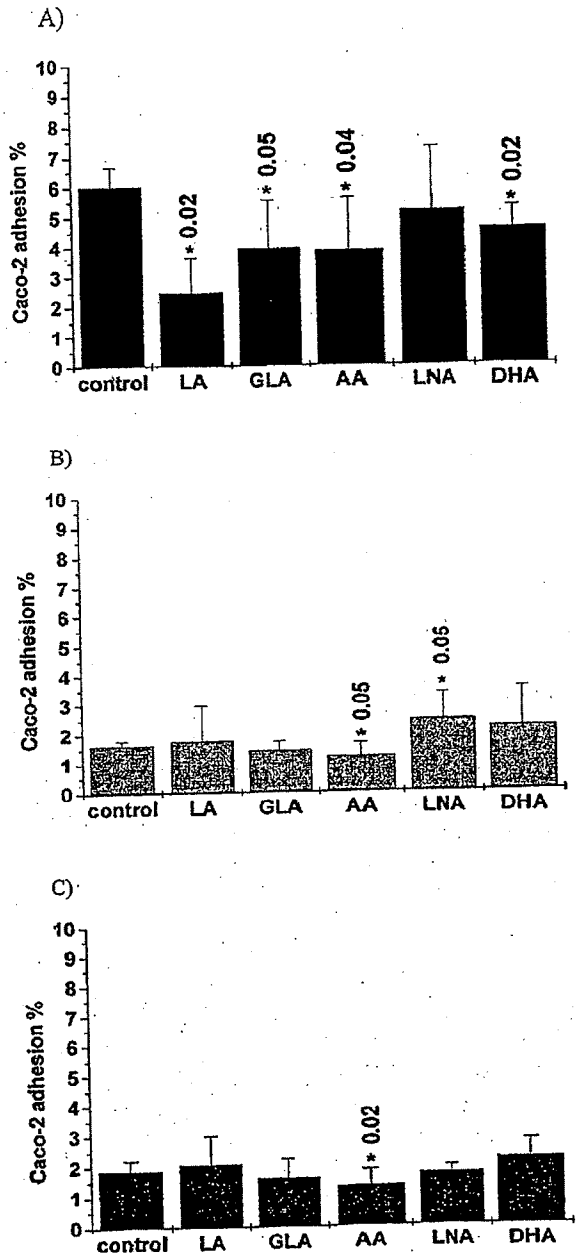

Fig. 1. Adhesion of *Lactobacillus* GG (A), *L. casei* Shirota (B) and *L. bulgaricus* (C) to human intestinal epithelial Caco-2 cell monolayers cultured with 10 μg ml^{-1} of linoleic, γ-linolenic, arachidonic, α-linolenic or docosahexaenoic acid indicate statistically significant differences compared to the unsupplemented medium. Abbreviations; linoleic acid (LA), γ-linolenic acid (GLA), arachidonic acid (AA), α-linolenic acid (LNA) and docosahexaenoic acid (DHA).

the adhesion of *L. casei* Shirota ($P < 0.01$ and $P < 0.03$, respectively) and *L. bulgaricus* ($P < 0.05$ and $P < 0.01$, respectively), whereas the adhesion of *Lactobacillus* GG was suppressed by these PUFA only at 40 μg ml^{-1} ($P < 0.01$

Table 2
The effect of different PUFA on bacterial growth and on adhesion to mucus isolated from healthy infants' faeces

PUFA	μg/ml	*Lactobacillus* GG		*L. casei* Shirota		*L. bulgaricus*	
		number of adhered bacteria (mean (S.D.))	% adhesion	number of adhered bacteria (mean (S.D.))	% adhesion	number of adhered bacteria (mean (S.D.))	% adhesion
Control	0	4.7E+6 (7.9E+5)	15.8	3.4E+6 (3.4E+6)	2.8	1.1E+7 (3.9E+5)	16.1
Linoleic acid (18:2 ω-6)	10	3.7E+6 (6.0E+5)	12.4	2.4E+6 (1.1E+6)	2.6	8.9E+6 (1.3E+6)	13.0
	20	3.1E+6 (1.7E+5)	10.3	6.3E+6 (5.2E+6)	7.0	9.5E+6 (4.8E+6)	13.9
	40	3.7E+6 (1.5E+6)	12.5	7.0E+5 (5.4E+5)	0.8[a]	1.0E+7 (6.3E+6)	15.2
γ-Linolenic acid (18:3 ω-6)	10	3.3E+6 (6.2E+5)	11.0	2.0E+6 (1.4E+6)	2.2	1.2E+7 (6.6E+5)	16.9
	20	3.5E+6 (7.5E+5)	11.6	1.5E+5 (3.2E+4)	0.2[a]	4.9E+6 (2.1E+6)	7.2[a]
	40	9.8E+5 (1.3E+5)	3.3[a]	1.6E+5 (9.5E+4)	0.2[a]	8.9E+5 (3.7E+5)	1.3[a]
Arachidonic acid (20:4 ω-6)	10	4.6E+6 (1.5E+6)	15.5	1.5E+7 (7.6E+6)	16.2[a]	8.2E+6 (1.1E+6)	12.0
	20	4.4E+6 (1.5E+6)	14.6	1.2E+5 (6.3E+4)	0.1[a]	4.7E+6 (3.3E+6)	6.9[a]
	40	1.5E+6 (8.1E+5)	5.1[a]	1.6E+5 (7.3E+4)	0.2[a]	6.7E+6 (2.9E+6)	9.8[a]
α-Linolenic acid (18:3 ω-3)	10	3.9E+6 (1.1E+6)	13.2	5.0E+6 (4.0E+6)	5.6	9.8E+6 (2.0E+6)	14.3
	20	3.0E+6 (1.0E+6)	10.0[a]	1.6E+6 (1.1E+6)	1.8	9.9E+6 (4.9E+6)	14.5
	40	9.2E+5 (7.1E+5)	3.1[a]	1.9E+5 (1.9E+5)	0.2[a]	2.3E+6 (1.8E+6)	3.4[a]
Docosahexaenoic acid (22:6 ω-3)	10	4.7E+6 (1.1E+6)	15.7	3.5E+5 (3.5E+5)	0.4[a]	1.2E+7 (2.9E+6)	16.7
	20	3.0E+6 (1.2E+6)	9.9[a]	1.6E+5 (3.8E+4)	0.2[a]	6.4E+6 (2.9E+6)	9.4
	40	5.9E+5 (7.6E+5)	2.0[a]	1.5E+5 (4.9E+4)	0.2[a]	5.6E+5 (1.1E+5)	0.8[a]

For mucus adhesion assay, bacterial concentrations were adjusted to optical density of 0.25 ± 0.5; therefore approximately 2.9E+7 *Lactobacillus* GG, 9.0E+7 *L. casei* Shirota and 6.9E+7 *L. bulgaricus* were added to mucus adhesion.
[a]Significantly different from control (Student's *t*-test, $P < 0.05$).

for both). Yet, the adhesion of *L. casei* Shirota was markedly promoted by arachidonic acid at 10 μg ml^{-1} in growth media ($P < 0.05$). The presence of α-linolenic acid in growth media inhibited the adhesion of *Lactobacillus* GG at 20 μg ml^{-1} or higher ($P < 0.05$), whilst 40 μg ml^{-1} of α-linolenic acid was needed to inhibit the adhesion of *L. casei* Shirota ($P < 0.001$) and *L. bulgaricus* ($P < 0.01$). Docosahexaenoic acid suppressed the adhesion properties of *L. casei* Shirota at 10 μg ml^{-1} or higher ($P < 0.03$), that of *Lactobacillus* GG at 20 μg ml^{-1} or higher ($P < 0.01$), and that of *L. bulgaricus* at 40 μg ml^{-1} ($P < 0.02$).

3.3. Caco-2 cell adhesion

Intestinal epithelial cells were also affected by PUFAs, evinced by variation in bacterial adhesion to differentially cultured Caco-2 cells. Culturing of Caco-2 cells in the presence of linoleic acid, γ-linolenic acid, arachidonic acid and docosahexaenoic acid reduced the adhesion of *Lactobacillus* GG (Fig. 1A). *L. casei* Shirota and *L. bulgaricus* adhered less on Caco-2 cells grown in the presence of arachidonic acid (Fig. 1B,C, respectively). However, Caco-2 cells grown in the presence of α-linolenic acid were able serve more adhesion sites for *L. casei* Shirota compared to control (Fig. 1B).

4. Discussion

The bactericidal activity of PUFA has been recognised for some time [7,12]. In particular, the antibacterial activity of free linoleic acid on lactic acid bacteria has been demonstrated [15,16]. In accordance, we have demonstrated here that free linoleic acid inhibited growth of *L. casei* Shirota and *L. bulgaricus* at high concentrations (40 μg ml^{-1}), but had no statistically significant effect on the growth of *Lactobacillus* GG. Also, the other free fatty acids tested were antibacterial, though γ-linolenic acid at a low dose promoted the growth of *L. casei* Shirota. As the flow cytometric viability assessment revealed that bacterial viability was not compromised by free PUFA, it is suggested that PUFA are not lethal to lactic acid bacteria but hinder the normal bacterial cell cycle.

Mucus adhesion has been considered important for persistence and subsequent immunological functions of probiotics [2]. *Lactobacillus* GG has been previously shown in vitro to adhere well to mucus [9], and the present study confirms these results. In addition, we have shown here that free PUFA could influence mucus adhesion properties of lactic acid bacteria. Bacterial mucus adhesion was inhibited by different free PUFA at 10–40 μg ml^{-1} in growth media, the only exception being arachidonic acid (10 μg ml^{-1}) that promoted mucus adhesion of *L. casei* Shirota. As mucus adhesion is the first step in persistence, the inhibited mucus adhesion shown here might reduce the number of bacteria able to adhere to epithelial cells, a key step for the health promoting functions of probiotics.

The influence of PUFA on intestinal epithelial cells have been demonstrated. Especially, ω-6 fatty acids have been shown to up-regulate epithelial permeability and inflammation associated with mucosal damage [17]. They have also been demonstrated to reduce the numbers of lactic acid bacteria in the digestive tract, whereas ω-3 fatty acids promoted the establishment of lactic acid bacteria in fish [18]. These authors suggested that PUFA could modify

adhesion sites for gastrointestinal micro-organisms by changing membrane fatty acid composition of the intestinal epithelial cells. Though the effects of free PUFA seen in our study may be dose-dependent, our results support their conclusion. Culturing of Caco-2 cells with arachidonic acid (ω-6 PUFA) reduced the Caco-2 cell adhesion of lactic acid bacteria, whereas α-linolenic acid, the major ω-3 PUFA, did not interfere with Caco-2 cell adhesion of *Lactobacillus* GG or *L. bulgaricus*, and even promoted the adhesion of *L. casei* Shirota. Thus, ω-6 fatty acids may exhibit multiple aspects in impairment of the intestinal barrier functions.

Probiotics are commonly introduced in specific carriers, such as fermented dairy products. These carriers contain approximately 1 μg ml^{-1} free PUFA, but due to the action of lipases, this concentration of free PUFA may exceed even 400 μg ml^{-1} [12]. However, this PUFA level can be considered as an overestimate as the secretions in the intestine increase the food bolus (dilution) and the absorption of fatty acid is a rapid process. Yet, as seen in this study, physiologically relevant levels of free PUFA may influence the functions of probiotics. Consequently, non-adhered probiotics may be 'washed out' from the gastrointestinal tract, and potential health benefits may be compromised. Thus, a better understanding of interactions between dietary factors, such as PUFA, and intestinal microflora is a prerequisite, when the beneficial effects of novel functional foods containing probiotics are designed and clinically assessed.

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References

[1] Björksten, B., Naaber, P., Sepp, E. and Mikelsaar, M. (1999) The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin. Exp. Allergy 29, 342–346.

[2] Salminen, S., Bouley, C., Boutron-Ruault, M.-C., Cummings, J.H., Franck, A., Gibson, G.R., Isolauri, E., Moreau, M.-C., Roberfroid, M. and Rowland, I. (1998) Functional food science and gastrointestinal physiology and function. Br. J. Nutr. 80 (S1), 147–171.

[3] Saavedra, J.M., Bauman, N.A., Oung, I., Perman, J.A. and Yolken, R.H. (1994) Feeding of *Bifidobacterium bifidum* and *Streptococcus thermophilus* to infants in hospital for prevention of diarrhoea and shedding of rotavirus. Lancet 344, 1046–1049.

[4] Isolauri, E., Juntunen, M., Rautanen, T., Sillanaukee, P. and Koivula, T. (1989) A human *Lactobacillus* strain (*Lactobacillus* GG) promotes recovery from acute diarrhea in children. Pediatrics 88, 90–97.

[5] Ouwehand, A., Kirjavainen, P., Shortt, C. and Salminen, S. (1999) Probiotics: mechanisms and established effects. Int. Dairy J. 9, 43–52.

[6] Uauy, R., Mena, P. and Rojas, C. (2000) Essential fatty acids in early life: structural and functional role. Proc. Nutr. Soc. 59, 3–15.

[7] Kodicek, E. and Worden, A.N. (1945) The effect of unsaturated fatty acids on *Lactobacillus helveticus* and other Gram-positive micro-organisms. Biochem. J. 39, 78–82.

[8] Goldin, B.R., Gorbach, S.L., Saxelin, M., Barakat, S., Gualtieri, L. and Salminen, S. (1992) Survival of *Lactobacillus* species (strain GG) in human gastrointestinal tract. Dig. Dis. Sci. 37, 121–128.

[9] Kirjavainen, P.V., Ouwehand, A.C., Isolauri, E. and Salminen, S.J. (1998) The ability of probiotic bacteria to bind to human intestinal mucus. FEMS Microbiol. Lett. 167, 185–189.

[10] Tuomola, E.M. and Salminen, S.J. (1998) Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int. J. Food Microbiol. 41, 45–51.

[11] de Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. J. Appl. Bact. 23, 130–135.

[12] Kabara, J.J., Swieczkowski, D.M., Conley, A.J. and Truant, J.P. (1972) Fatty acids and derivatives as antimicrobial agents. Antimicrob. Agents Chemother. 2, 23–28.

[13] Chambon, M. (1996) Milk Fat. In: Oil and Fats Manual (Karleskind, A., Ed.), vol. 1, pp. 277–286. Lavoisier Publishing, France.

[14] Virta, M., Lineri, S., Kankaanpää, P., Karp, M., Peltonen, K., Nuutila, J. and Lilius, E.-M. (1998) Determination of complement-mediated killing of bacteria by viability staining and bioluminescence. Appl. Environ. Microbiol. 64, 515–519.

[15] Boyaval, P., Corre, C., Dupuis, C. and Roussel, E. (1995) Effects of free acids on propionic acid bacteria. Lait 75, 17–29.

[16] Jiang, J., Björck, L. and Fonden, R. (1998) Production of conjugated linoleic acid by dairy starter cultures. J. Appl. Microbiol. 85, 95–102.

[17] Ohtsuka, Y., Yamashiro, Y., Shimizu, T., Nagata, S., Igarashi, J., Shinohara, K., Oguchi, S. and Yabuta, K. (1997) Reducing cell membrane *n*-6 fatty acids attenuate mucosal damage in food-sensitive enteropathy in mice. Pediatr. Res. 42, 835–839.

[18] Ringø, E., Bendiksen, H.R., Gausen, S.J., Sundsfjord, A. and Olsen, R.E. (1998) The effect of dietary fatty acids on lactic acid bacteria associated with the epithelial mucosa and form faecalia of Arctic charr, *Salvelinus alpinus* (L.). J. Appl. Microbiol. 85, 855–864.